

OB PROTEIN COMPOSITIONS AND METHODS

*Insert a.7*

Field of the Invention

5           The present invention relates to OB protein compositions and methods for preparation and use thereof.

Background

10           Although the molecular basis for obesity is largely unknown, the identification of the "OB gene" and protein encoded by ("OB protein") has shed some light on mechanisms the body uses to regulate body fat deposition. Zhang et al., Nature 372: 425-432 (1994);  
15   see also, the Correction at Nature 374: 479 (1995). The OB protein has been demonstrated to be active in vivo in both ob/ob mutant mice (mice obese due to a defect in the production of the OB gene product) as well as in normal, wild type mice. The biological activity  
20   manifests itself in, among other things, weight loss. To date, however, optimum conditions for obtaining the rapid weight loss in normal animals has not been ascertained. In fact, some studies have shown that, when administered by injection, rather large dosages (10  
25   mg of recombinant murine protein/kg body weight/day) are necessary for normal mice to lose 2.6% of their body weight (at the end of a 32 day period). While presently uncertain, one explanation for the necessity of such large dosages is that the optimum weight loss effects  
30   are seen predominantly when the protein is in constant circulation, a condition that may not be efficiently achieved by injecting the protein.

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Summary of the Invention

The present invention stems from the observation that, as compared to administering OB protein by injection, administering OB protein by continuous pump infusion results in equivalent (or better) weight loss, in a shorter time, and with substantially lower dosages. The working example below demonstrates that a dose of 0.5 mg protein/kg body weight/day, administered via implantable osmotic pump, results in a weight loss of over 4% (as compared to baseline weight). This is in substantial contrast to other studies where similar, or less weight loss (at a comparable time point) was observed with intraperitoneal injection at the relatively high dosage of 10 mg of protein/kg body weight/day.

Thus, one aspect of the present invention is a method of treating excess weight by administering OB protein in a form for constant supply, at a dosage of less than or equal to about 1 mg protein/kg body weight/day. The dosage of less than or equal to about 1 mg protein/kg/day refers to dosages sufficient to result in observable weight loss. This is apparent from the present studies where a dosage of 0.5 mg/kg/day was sufficient to result in observable weight loss when continuously administered. In studies where injection had been the mode of administration, far higher dosages were required for weight loss. At injection dosages of 0.1 and 1 mg/kg/day, substantially no weight loss was observed in wild type (normal) mice. For example, in one study, at a comparable time point (6th day), there was a .2% loss at the 1 mg/kg dose (data not shown). Minimal weight loss was observed at the relatively high 10 mg/kg/day dose. (1.9% weight loss at day 6, data not shown). Thus, the present invention provides for dosages of 1 mg/kg/day or less when administered so that the supply of protein is continuous.

Connected with the present studies are the compositions and methods used for production of recombinant murine and human OB protein. The first example below discloses the preparation of recombinant murine protein, and the second example below discloses the preparation of recombinant human protein.

Additional aspects of the present invention, therefore, include the below compositions and methods for preparing recombinant murine methionyl OB protein and recombinant human methionyl OB protein, including DNA sequences, vectors, host cells, methods of fermentation, and methods of purification.

#### Detailed Description

The present invention stems from the observation that continuous administration of OB protein results in the need for much lower dosages for weight loss than those dosages required by acute daily injection. As set forth above, a dosage of 1 mg protein/kg body weight/day or less, continuously administered, resulted in rapid weight loss. When the underivatized protein was administered by acute injection at the 1 mg/kg/day dose, almost no weight loss in wild type (normal) mice occurred.

The OB protein may be selected from the recombinant murine and human methionyl proteins set forth below (SEQ. ID Nos. <sup>3</sup>~~2~~ and <sup>6</sup>~~4~~) or those lacking a glutaminy residue at position 28. (See Zhang et al, Nature, supra, at page 428.) The recombinant human OB gene product is, as a mature protein, 146 amino acids; some of the DNAs obtained were observed to encode a protein lacking a glutamine residue at position 28. Zhang et al., Nature 372 at 428. The murine protein is substantially homologous to the human protein, particularly as a mature protein, and, further, particularly at the N-terminus. One may prepare an

analog of the recombinant human protein by altering (such as substituting amino acid residues), in the recombinant human sequence, the amino acids which diverge from the murine sequence. Because the recombinant human protein has biological activity in mice, such analog would likely be active. Proteins lacking an N-terminal methionyl residue, such as those produced by eukaryotic expression, are also available for use.

In addition, although the present working example involved continuous administration via implantable pump, it is contemplated that other modes of continuous administration may be practiced. For example, chemical derivatization may result in sustained release forms of the protein which have the effect of continuous presence in the blood stream, in predictable amounts. Thus, one may derivatize the above proteins to effectuate such continuous administration. The dosage of 1 mg protein/kg body weight/day or less herein refers to the mass of protein, exclusive of other chemical moieties used to derivatize the protein.

Generally, the present protein (herein the term "protein" is used to include "peptide", unless otherwise indicated) may be derivatized by the attachment of one or more chemical moieties to the protein moiety. The chemically modified derivatives may be further formulated for intraarterial, intraperitoneal, intramuscular subcutaneous, intravenous, oral, nasal, pulmonary, topical or other routes of administration. Chemical modification of biologically active proteins has been found to provide additional advantages under certain circumstances, such as increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. See U.S. Patent No. 4,179,337, Davis et al., issued December 18, 1979. For a review, see Abuchowski et al., in

The chemical moieties suitable for derivatization may be selected from among water soluble polymers. The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For the present proteins and peptides, the effectiveness of the derivatization may be ascertained by administering the derivative, in the desired form (i.e., by osmotic pump, or, more preferably, by injection or infusion, or, further formulated for oral, pulmonary or nasal delivery, for example), and measuring weight loss.

The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol,

carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random  
5 copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, propylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol. Polyethylene glycol propionaldehyde may have  
10 advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about  
15 2kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic  
20 profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or  
25 some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as  
30 different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in  
35 terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined

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by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

5           The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art. E.g., EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20: 1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

30           One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation

reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pK<sub>a</sub> differences between the ε-amino group of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

In yet another aspect of the present invention, provided are methods of using pharmaceutical compositions of the proteins and derivatives. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal or other forms of administration which allow for the desired



circulating dose of about 1 mg protein/kg body weight/day or less. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of protein or derivative products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. The effective amounts are those herein described.

The OB proteins and derivatives described are useful for modulation of the rate or quantity of fat cell deposition in a mammal. This is thought to be accomplished, in part, by a reduction in appetite, i.e., a reduction in food intake. Thus, one observable result is weight loss, or, put another way, a method of treating excess weight (via weight loss). Thus, the present compositions are useful for the manufacture of a medicament for treating excess weight in a mammal.

Another aspect is a method for reducing appetite. Either of these aspects, modulation of fat deposition or modulation of appetite, are particularly important treatments for humans (or other mammals) who desire to  
5 lose weight.

One skilled in the art will be able to ascertain other effective dosages by administration and observing weight loss. Here, the dosage of 1 mg protein/kg body weight/day or less was seen to be  
10 particularly effective, when administered on a continuous basis. More particularly, the dosage of 0.5 mg/kg body weight/day was seen to be particularly effective on normal mice. Excess weight refers to body mass for which removal is desired. It is contemplated  
15 that the present compositions and methods will be used to treat cases where removal of such excess weight (as a result of the present invention) will benefit other health concerns, such as diabetes, high blood pressure or cardiac problems, high cholesterol levels, low  
20 locomotion levels and other manifestations of excess weight. As such, the present compositions and methods may be used in conjunction with other medicaments, such as those useful for the treatment of diabetes (e.g., insulin, and possibly amylin), cholesterol and blood  
25 pressure lowering medicaments, and locomotion increasing medicaments (e.g., amphetamines). Such administration may be simultaneous or may be in serriatim.

In addition, the present compositions and methods may be used in conjunction with surgical  
30 procedures, such as cosmetic surgeries designed to alter the overall appearance of a body (e.g., liposuction or laser surgeries designed to reduce body mass). The health benefits of cardiac surgeries may be increased with concomitant use of the present compositions and  
35 methods.

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Therefore, the present invention encompasses a method of treating excess weight in a mammal by continuous administration of 1 mg protein/kg body weight/day or less of an OB protein selected from the group consisting of:

(a) recombinant methionyl murine OB protein (~~SEQ. ID No. 2~~);

(b) recombinant methionyl human OB protein (~~SEQ ID No. 1~~);

(c) the protein of (a) or (b) lacking the methionyl residue at position -1;

(d) the protein of (a), (b) or (c) lacking a glutamine at position 28; and

(e) a chemically modified derivative of (a), (b), (c) or (d), wherein the chemical modification allows for an increase in circulation time.

Preferably, the composition of subpart (e) is a pegylated derivative, and, more preferably, an N-terminally pegylated derivative.

The derivative of subpart (e) allows for continuous administration of the protein by increasing the circulation time of the (unmodified) protein. The present invention also encompasses a method of treating excess weight where the method of continuous administration is by implantable pump, such as an osmotic pump.

In other aspects, the present invention relates to recombinant murine and recombinant human OB DNAs and proteins, such as those of SEQ. ID NOs. 1, 2, 3, <sup>4, 5, and 6</sup> ~~and 4~~, below. The recombinant proteins below are bacterially expressed, and contain N-terminal methionyl residues. Vectors and host cells useful for producing such proteins are also provided. The vectors include pCFM1656 containing SEQ ID No. 1 or <sup>4</sup> ~~3~~, and host cells containing such vectors.

Methods for preparation of the recombinant proteins are also provided, including methods for fermentation and methods for purification.

In particular, the use of sarcosine for  
5 refolding of OB protein in solution, obtained from  
bacterial inclusion bodies, provided for extremely  
efficient refolding. When proteins are expressed in  
bacteria, they may not be in the proper three-  
dimensional configuration, or, as referred to herein,  
10 properly refolded. The three dimensional configuration  
may be critical for biological activity, and storage  
stability. Although Sarkosyl has been used in processes  
for purification of another protein (G-CSF, e.g., WO  
89/10932), surprisingly, the use of sarcosine for the OB  
15 protein has resulted in a refolding efficiency of over  
95%. Contemplated herein is the use of N-  
lauroylsarcosine in a range of 0.5% - 2.0 % weight per  
volume of OB protein in solution (obtained from  
inclusion bodies). With the use of 1% sodium sarcosine,  
20 the refolding efficiency, as determined by SDS PAGE and  
reverse phase HPLC, was 95% or greater. While one  
skilled in the art will recognize that other  
compositions may be used for refolding, the use of  
N-lauroyl sarcosine, as illustrated in the examples  
25 below, is particularly advantageous for providing  
extremely efficient refolding. The removal of sarcosine  
was accomplished using Dowex®.

Therefore, the present invention also includes  
a method of refolding partially purified OB protein in a  
30 solution obtained from inclusion bodies, said partially  
purified OB protein selected from the group consisting  
of:

- (a) recombinant methionyl murine OB protein  
(SEQ. ID. No. 2);
- 35 (b) recombinant methionyl human OB protein  
(~~SEQ ID No. 1~~);

(c) the protein of (a) or (b) lacking the methionyl residue at position -1;

wherein said refolding is accomplished using sarcosine.

5           The present invention also includes methods of wherein said N-lauroyl sarcosine is used at a concentration of 0.5% - 2.0% weight per volume of solution, and, more particularly, the use of 1% N-lauroyl sarcosine. An oxidizing agent, such as copper  
10       sulfate, is also used in the refolding process.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

15           EXAMPLE 1: Use of Murine OB Protein in a Continuous Pump Infusion System

This example demonstrates that continuous infusion of OB protein results in weight loss in normal mice. Normal (non-obese) mice were administered murine  
20       OB protein via osmotic pump infusion. A dosage of 0.5 mg protein/kg body weight/day resulted in a 4.62% (+/- 1.34%) loss from baseline weight by the 6th day of infusion.

25       MATERIALS AND METHODS

Animals: Wild type (+/+) C57B16 mice were used for this experiment. The age of the mice at the initial time point was 8 weeks, and the animals were weight  
30       stabilized. 10 mice were used for each cohort (vehicle vs. protein).

Animal Handling.

Feeding and weight measurement. Mice were given  
35       ground rodent chow (PMI Feeds, Inc.) in powdered food feeders (Allentown Caging and Equipment) which allowed a

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more accurate and sensitive measurement than use of regular block chow. Weight was measured at the same time each day (2:00 p.m.), for a period of 6 days. Body weight on the day prior to the infusion was defined as  
5 baseline weight. The mice used weighed 18-22 grams.

Housing. Mice were single-housed, and maintained under humane conditions.

10 Administration of Protein or Vehicle. Protein (as described below) or vehicle (phosphate buffered saline, pH 7.4) were administered by osmotic pump infusion. Alzet osmotic minipumps (Alza, Palo Alto, CA, model no. 1007D) were surgically placed in each mice in a  
15 subcutaneous pocket in the subscapular area . The pumps were calibrated to administer 0.5  $\mu$ l protein in solution per hour for a dosage of 0.5 mg protein/kg body weight/day.

20 Controls: Control animals were those who had a Alzet osmotic minipump infusing phosphate buffered saline (pH 7.4) .

Protein: Recombinant murine OB protein was used for  
25 the present experiments, generally at a concentration of about 0.9 mg/ml phosphate buffered saline, pH 7.4. The amino acid sequence (and DNA sequence) used was the following:

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|    |     |  |     |
|----|-----|--|-----|
| 5  | 9   | TCTAGATTGTTAGTTTAACTTTTAGAAGGAGGAATAACATATGGTACCGATCCAGAAAGT<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>AGATCTAAACTCAAATTTGAAAATCTTCCTCCTTATTGTATACCATGGCTAGGTCTTTCA<br>M V P I Q K V -                            | 68  |
| 10 | 69  | TCAGGACGACACCAAAACCTTAATTAAACGATCGTTACGCGTATCAACGACATCAGTCA<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>AGTCCTGCTGTGGTTTTGAATTAATTTTGTAGCAATGCGCATAGTTGCTGTAGTCAGT<br>Q D D T K T L I K T I V T R I N D I S H -     | 128 |
| 15 | 129 | CACCCAGTCGGTCTCCGCTAAACAGCGTGTTACCGGTCTGGACTTCATCCCGGGTCTGCA<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>GTGGGTCAGCCAGAGGCGATTTGTGCGACAATGGCCAGACCTGAAGTAGGGCCCAGACGT<br>T Q S V S A K Q R V T G L D F I P G L H -  | 188 |
| 20 | 189 | CCCGATCCTAAGCTTGTCCAAAATGGACCAGACCCTGGCTGTATACCAGCAGGTGTTAAC<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>GGGCTAGGATTTCGAACAGGTTTTACCTGGTCTGGGACCGACATATGGTCGTCCACAATTG<br>P I L S L S K M D Q T L A V Y Q Q V L T - | 248 |
| 25 | 249 | CTCCCTGCCGTCCCAGAACGTTCTTCAGATCGCTAACGACCTCGAGAACCTTCGCGACCT<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>GAGGGACGGCAGGGTCTTGCAAGAAGTCTAGCGATTGCTGGAGCTCTTGGAAGCGCTGGA<br>S L P S Q N V L Q I A N D L E N L R D L -  | 308 |
| 30 | 309 | GCTGCACCTGCTGGCATTCTCCAAATCCTGCTCCCTGCCGCAGACCTCAGGTCTTCAGAA<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>CGACGTGGACGACCGTAAGAGGTTTAGGACGAGGGACGGCGTCTGGAGTCCAGAAGTCTT<br>L H L L A F S K S C S L P Q T S G L Q K -  | 368 |
| 35 | 369 | ACCGGAATCCCTGGACGGGGTCTTGAAGCATCCCTGTACAGCACCAGAGTTGTTGCTCT<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>TGGCCTTAGGGACCTGCCCCAGGACCTTCGTAGGGACATGTCGTGGCTTCAACAACGAGA<br>P E S L D G V L E A S L Y S T E V V A L -   | 428 |
| 40 | 429 | GTCCCGTCTGCAGGGTTCCCTTCAGGACATCCTTCAGCAGCTGGACGTTTCTCCGGAATG<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>CAGGGCAGACGTCCCAAGGGAAGTCCTGTAGGAAGTCGTCGACCTGCAAAGAGGCCTTAC<br>S R L Q G S L Q D I L Q Q L D V S P E C -  | 488 |
| 45 | 489 | TTAATGGATCC<br>-+-----+-----+-----+-----+-----+-----+-----+-----+<br>AATTACCTAGG   |     |

Herein, the first amino acid of the amino acid sequence for recombinant protein is referred to as +1, and is valine, and the amino acid at position -1 is methionine. The C-terminal amino acid is number 146 (cysteine).

The cloning of the murine OB DNA for expression in E. coli was done as follows. The DNA sequence was deduced from the published peptide sequence that appeared in Zhang et al., Nature 372:425-432 (1994). It was reverse translated using E. coli optimal codons. The terminal cloning sites were XbaI to BamHI. A ribosomal binding enhancer and a strong ribosomal binding site were included in front of the coding region. The duplex DNA sequence was synthesized using standard techniques. Correct clones were confirmed by demonstrating expression of the recombinant protein and presence of the correct OB DNA sequence in the resident plasmid.

#### Expression Vector and Host Strain

The plasmid expression vector used was pCFM1656, ATCC Accession No. 69576. The above DNA was ligated into the expression vector pCFM1656 which had been linearized with XbaI and BamHI and transformed into the E. coli host strain, FM5. E. coli FM5 cells were derived at Amgen Inc., Thousand Oaks, CA from E. coli K-12 strain (Bachmann, et al., Bacteriol. Rev. 40: 116-167 (1976)) and contain the integrated lambda phage repressor gene, cI<sub>857</sub> (Sussman et al., C.R. Acad. Sci. 254: 1517-1579 (1962)). Vector production, cell transformation, and colony selection were performed by standard methods. E.g., Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2d Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Host cells were grown in LB media.

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Fermentation Process A three-phase fermentation protocol was used known as a fed-batch process. Media compositions are set forth below.

5                   Batch: A nitrogen and phosphate source were  
sterilized (by raising to 122 °C for 35 minutes, 18-20  
psi) in the fermentation vessel (Biolafitte, 12 liter  
capacity). Upon cooling, carbon, magnesium, vitamin,  
and trace metal sources were added aseptically. An  
10 overnight culture of the above recombinant murine  
protein-producing bacteria (16 hours or more) of 500 mL  
(grown in LB broth) was added to the fermentor.

                  Feed I: Upon reaching between 4.0-6.0 OD<sub>600</sub>,  
15 cultures were fed with Feed I. The glucose was fed at a  
limiting rate in order to control the growth rate ( $\mu$ ) .  
An automated system (called the Distributive Control  
System) was instructed to control the growth rate to  
0.15 generations per hour.

20                   Feed II: When the OD<sub>600</sub> had reached 30,  
culture temperature was slowly increased to 42°C and the  
feed was changed to Feed II, below. The fermentation was  
then allowed to continue for 10 hours with sampling  
25 every 2 hours. After 10 hours, the contents of the  
fermentor was chilled to below 20°C and harvested by  
centrifugation.

Media Composition:

|    |          |          |   |
|----|----------|----------|---|
| 5  | Batch:   | 10 g/L   | Yeast extract                                   |
|    |          | 5.25 g/L | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> |
|    |          | 3.5 g/L  | K <sub>2</sub> HPO <sub>4</sub>                 |
|    |          | 4.0 g/L  | KH <sub>2</sub> PO <sub>4</sub>                 |
|    |          | 5.0 g/L  | Glucose   |
|    |          | 1.0 g/L  | MgSO <sub>4</sub> ·7H <sub>2</sub> O            |
|    |          | 2.0 mL/L | Vitamin Solution                                |
|    |          | 2.0 mL/L | Trace Metal Solution                            |
| 10 |          | 1.0 mL/L | P2000 Antifoam                                  |
|    |          |          |   |
| 15 | Feed I:  | 50 g/L   | Bacto-tryptone                                  |
|    |          | 50 g/L   | Yeast extract                                   |
|    |          | 450 g/L  | Glucose   |
|    |          | 8.75 g/L | MgSO <sub>4</sub> ·7H <sub>2</sub> O            |
|    |          | 10 mL/L  | Vitamin Solution                                |
|    |          | 10 mL/L  | Trace Metal Solution                            |
|    |          |          |   |
|    |          |          |   |
| 20 | Feed II: | 200 g/L  | Bacto-tryptone                                  |
|    |          | 100 g/L  | Yeast extract                                   |
|    |          | 110 g/L  | Glucose   |

Vitamin Solution (Batch and Feed I):

0.5 g Biotin, 0.4 g Folic acid, and 4.2 g riboflavin, were dissolved in 450 mls H<sub>2</sub>O and 3 mls 10 N NaOH, and brought to 500 mls in H<sub>2</sub>O. 14 g pyridoxine-HCl and 61 g niacin were dissolved 150 ml H<sub>2</sub>O and 50 ml 10 N NaOH, and brought to 250 ml in H<sub>2</sub>O. 54 g pantothenic acid was dissolved in 200 ml H<sub>2</sub>O, and brought to 250 ml. The three solutions were combined and brought to 10 liters total volume.

Trace Metal Solution (Batch and Feed I):

Ferric Chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ): 27 g/L

Zinc Chloride ( $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$ ): 2 g/L

Cobalt Chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ): 2 g/L

5 Sodium Molybdate ( $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ): 2 g/L

Calcium Chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ): 1 g/L

Cupric Sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ): 1.9 g/L

Boric Acid ( $\text{H}_3\text{BO}_3$ ): 0.5 g/L

Manganese Chloride ( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ): 1.6 g/L

10 Sodium Citrate dihydrate: 73.5 g/L

#### Purification Process for Murine OB Protein

Purification was accomplished by the following steps (unless otherwise noted, the following steps were performed at 4°C):

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1. Cell paste. E. coli cell paste was suspended in 5 times volume of 7 mM of EDTA, pH 7.0. The cells in the EDTA were further broken by two passes through a microfluidizer. The broken cells were centrifuged at 4.2 K rpm for 1 hour in a Beckman J6-B centrifuge with a JS-4.2 rotor.

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2. Inclusion body wash #1. The supernatant from above was removed, and the pellet was resuspended with 5 times volume of 7 mM EDTA, pH 7.0, and homogenized. This mixture was centrifuged as in step 1.

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3. Inclusion body wash #2. The supernatant from above was removed, and the pellet was resuspended in ten times volume of 20 mM tris, pH 8.5, 10 mM DTT, and 1% deoxycholate, and homogenized. This mixture was centrifuged as in step 1.

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4. Inclusion body wash #3. The supernatant from above was removed and the pellet was resuspended in ten times volume of distilled water, and homogenized. This mixture was centrifuged as in step 1.

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08420600 "082797"

5. Refolding. The pellet was refolded with 15 volumes of 10 mM HEPES, pH 8.5, 1% sodium sarcosine (N-lauroyl sarcosine), at room temperature. After 60 minutes, the solution is made to be 60  $\mu$ M copper sulfate, and then stirred overnight.

6. Removal of sarcosine. The refolding mixture was diluted with 5 volumes of 10 mM tris buffer, pH 7.5, and centrifuged as in step 1. The supernatant was collected, and mixed with agitation for one hour with Dowex® 1-X4 resin (Dow Chemical Co., Midland MI), 20-50 mesh, chloride form, at 0.066% total volume of diluted refolding mix. See WO 89/10932 at page 26 for more information on Dowex®. This mixture was poured into a column and the eluant was collected. Removal of sarcosine was ascertained by reverse phase HPLC.

7. Acid precipitation. The eluant from the previous step was collected, and pH adjusted to pH 5.5, and incubated for 30 minutes at room temperature. This mixture was centrifuged as in step 1.

8. Cation exchange chromatography. The pH of the supernatant from the previous step was adjusted to pH 4.2, and loaded on CM Sepharose Fast Flow (at 7% volume). 20 column volumes of salt gradient were done at 20 mM NaOAC, pH 4.2, 0 M to 1.0 M NaCl.

9. Hydrophobic interaction chromatography. The CM Sepharose pool of peak fractions (ascertained from ultraviolet absorbance) from the above step was made to be 0.2 M ammonium sulfate. A 20 column volume reverse salt gradient was done at 5 mM NaOAC, pH 4.2, with .4 M to 0 M ammonium sulfate. This material was concentrated and diafiltered into PBS.

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# Results

Presented below are the percent (%) differences from baseline weight in C57Bl6J mice (8 weeks old) :

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Table 1: Weight Loss Upon Continuous Infusion

| <u>Time (days)</u> | <u>Vehicle (PBS)</u> | <u>Recombinant OB protein</u> |
|--------------------|----------------------|-------------------------------|
| Days 1-2           | 3.24 +/- 1.13        | 1.68 +/- 1.4                  |
| Days 3-4           | 4.3 +/- .97          | -2.12 +/- .79                 |
| Days 5-6           | 4.64 +/- .96         | -4.62 +/- 1.3                 |

As can be seen, at the end of a 6 day continuous infusion regime, animals receiving the OB protein lost over 4% of their body weight, as compared to baseline. This is a substantially more rapid weight loss than has been observed with intraperitoneal (i.p.) injection. Weight loss at the end of a 32-day injection period, in wild type (normal) mice, with daily i.p. injections of recombinant murine OB protein at a 10 mg/kg dose was 2.6%, and had not been more than 4% at any time during the dosing schedule (data not shown). The present data indicate that with continuous infusion, a 20-fold lower dosage (0.5 mg/kg vs. 10 mg/kg) achieves more weight loss in a shorter time period.

The results seen here are statistically significant, e.g., -4.62% with  $p < .0001$ .

EXAMPLE 2: Dose Response Studies

An additional study demonstrated that there was a dose response to continuous administration of OB protein. In this study, non-obese, CD-1 mice, weighing 35-40 g were administered recombinant murine OB protein using methods similar to the above example. The results are set forth in Table 2, below, (with % body weight lost as compared to baseline, measured as above):

Table 2: Dose Response With Continuous Administration

| Dose           | Time  | % Reduction in body weight |
|----------------|-------|----------------------------|
| 0.03 mg/kg/day | Day 2 | 3.5                        |
| 1 mg/kg/day    | Day 2 | 7.5                        |
| 1 mg/kg/day    | Day 4 | 14                         |

As can be seen, increasing the dose from 0.03 mg/kg/day to 1 mg/kg/day increased the weight lost from 3.5% to 7.5%. It is also noteworthy that at day 4, the 1 mg/kg/day dosage resulted in a 14% reduction in body weight.

EXAMPLE 3: Cloning and Expression of a Recombinant Human Methionyl OB Protein

This example provides compositions and methods for preparation of a recombinant human version of the OB protein.

The recombinant human version of the OB DNA was constructed from the murine OB DNA, as in Example 1, above, by replacing the region between the MluI and BamHI sites with duplex DNA (made from synthetic oligonucleotides) in which 20 codon substitutions had been designed. The MluI site is shown under the solid line in the sequence below. This DNA was put into the pCFM1656

1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2729 2730 2731 2732 2733 2734 2735 2736 2737 2738 2739 2740 2741 2742 2743 2744 2745 2746 2747 2748 2749 2750 2751 2752 2753 2754 2755 2756 2757 2758 2759 2760 2761 2762 2763 2764 2765 2766 2767 2768 2769 2770 2771 2772 2773 2774 2775 2776 2777 2778 2779 2780 2781 2782 2783 2784 2785 2786 2787 2788 2789 2790 2791 2792 2793 2794 2795 2796 2797 2798 2799 2800 2801 2802 2803 2804 2805 2806 2807 2

[illegible]

5 1 CATATGGTACCGATCCAGAAAAGTTTCAGGACGACACCAAAACCTTAATTAAACGATCGTT 60  
GTATACCATGGCTAGGTCTTTCAAGTCCTGCTGTGGTTTTGGAATTAATTTTGCTAGCAA  
M V P I Q K V Q D D T K T L I K T I V -

10 61 ACGCGTATCAACGACATCAGTCACACCCAGTCGGTGAGCTCTAAACAGCGTGTTACAGGC 120  
TGCGCATAGTTGCTGTAGTCAGTGTGGGTGAGCCACTCGAGATTTGTCGCACAATGTCCG  
T R I N D I S H T Q S V S S K Q R V T G -

15 121 CTGGACTTCATCCCGGTCTGCACCCGATCCTGACCTTGTCCTAAAATGGACCAGACCCTG 180  
GACCTGAAGTAGGGCCCAGACGTGGGCTAGGACTGGAACAGGTTTTTACCTGGTCTGGGAC  
L D F I P G L H P I L T L S K M D Q T L -

20 181 GCTGTATACCAGCAGATCTTAACCTCCATGCCGTCCCGTAACGTTCTTCAGATCTCTAAC 240  
CGACATATGGTCGTCTAGAATTGGAGGTACGGCAGGGCATTGCAAGAAGTCTAGAGATTG  
A V Y Q Q I L T S M P S R N V L Q I S N -

30 241 GACCTCGAGAACCTTCGCGACCTGCTGCACGTGCTGGCATTCTCCAAATCCTGCCACCTG 300  
CTGGAGCTCTTGGAAGCGCTGGACGACGTGCACGACCGTAAGAGGTTTAGGACGGTGGAC  
D L E N L R D L L H V L A F S K S C H L -

35 301 CCATGGGCTTCAGGTCTTGAGACTCTGGACTCTCTGGGCGGGGTCCTGGAAGCATCCGGT 360  
GGTACCCGAAGTCCAGAACTCTGAGACCTGAGAGACCCGCCCCAGGACCTTCGTAGGCCA  
P W A S G L E T L D S L G G V L E A S G -

40 361 TACAGCACCGAAGTTGTTGCTCTGTCCCGTCTGCAGGGTTCCCTTCAGGACATGCTTTGG 420  
ATGTCGTGGCTTCAACAACGAGACAGGGCAGACGTCCCAAGGGAAGTCCTGTACGAAACC  
Y S T E V V A L S R L Q G S L Q D M L W -

50 421 CAGCTGGACCTGTCTCCGGGTGTTAATGGATCC 454  
GTCGACCTGGACAGAGGCCCAACAATTACCTAGG  
Q L D L S P G C \*



Fermentation: Fermentation of the above host cells to produce recombinant human OB protein was accomplished using the conditions and compositions as described above for recombinant murine material. The results were analyzed for yield (grams ob DNA product/liter of fermentation broth), prior to purification of the recombinant human OB material. (Minor amounts of bacterial protein were present.) Bacterial expression was also calculated.

Table 3: Analysis of Recombinant Human OB Protein Expression

| Timepoint           | OD<br>(@600 nm) | Yield<br>(g/L) | Expression<br>(mg/OD·L) |
|---------------------|-----------------|----------------|-------------------------|
| Ind. + 2<br>hours.  | 47              | 1.91           | 41                      |
| Ind. + 4<br>hours.  | 79              | 9.48           | 120                     |
| Ind. + 6<br>hours.  | 95              | 13.01          | 137                     |
| Ind. + 8<br>hours.  | 94              | 13.24          | 141                     |
| Ind. + 10<br>hours. | 98              | 14.65          | 149                     |

abbreviations: Ind. + \_\_ hours means the hours after induction of protein expression, as described in Example I for the recombinant murine material using pCFM1656  
OD: optical density, as measured by spectrophotometer  
milligrams per OD unit per liter  
mg/OD·L: expression in terms of milligrams of protein per OD unit per liter.

g/L: grams protein/liter fermentation broth

Purification of the recombinant human OB

- protein: Recombinant human protein may be purified using methods similar to those used for purification of recombinant murine protein, as in Example 1, above. For
- 5 preparation of recombinant human OB protein, step 8 was performed by adjusting the pH of the supernatant from step 7 to pH 5.0, and loading this onto a CM Sepharose fast flow column. The 20 column volume salt gradient was performed at 20 mM NaOAC, pH 5.5, 0M to 0.5 M NaCl.
- 10 Step 9 was performed by diluting the CM Sepharose pool four fold with water, and adjusting the pH to 7.5. This mixture was made to 0.7 M ammonium sulfate. Twenty column volume reverse salt gradient was done at 5 mM NaOAC, pH 5.5, 0.2 M to 0M ammonium sulfate. Otherwise,
- 15 the above steps were identical.

- While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended
- 20 claims cover all such equivalent variations which come within the scope of the invention as claimed.